Habitually used hibernation sites of paper wasps are marked with venom and cuticular peptides

Habitually used hibernation sites of paper wasps are marked with venom and cuticular peptides

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Chemical communication is widespread in insects and is crucial for colony organization in social species [1]. The environment and the signal requirements influence the chemical characteristics of the pheromone employed [1]. Highly volatile substances elicit rapid responses, as in alarm signalling, whereas low volatility compounds provide long-lasting cues suited to territory marking and nest-mate recognition [1]. The social behaviour of *Polistes* paper wasps, from nest foundation to autumnal colony dissolution, has been extensively studied; however, little is known about the subsequent non-colonial phase. At the end of summer, the newly emerged mated queens leave their colonies and can form clusters of hundreds of individuals in sheltered places, known as hibernacula, where they spend the cold season (Figure 1A). Hibernation is a critical period and the selection of appropriate winter quarters is probably essential for survival [2]. Wasps often over-winter in the same shelter year after year, which suggests the existence of persistent cues that mark used hibernacula. This point was first made by Rau in 1930 [2], who also observed *Polistes annularis* females inspecting possible hibernacula [3]. We report here that, for future queens of *Polistes dominulus*, these persistent cues are peptides, found on their cuticle and in their venom [4], which act as hibernacula-marking pheromones from one generation to the next.

We performed simple preference bioassays in which single hibernating wasps could choose between two different test tubes for a place to continue their hibernation (Figure 1B; see Supplemental data available on-line with this issue for details of the Experimental procedures). We observed that wasps preferred tubes previously occupied by other wasps, even eight months before, over unused tubes (Table 1, experiments 1 and 2). They also preferred tubes treated with residues of methanol extracts from previously occupied tubes over tubes treated with methanol extracts from unused tubes (Table 1, experiment 3). In contrast, they did not prefer pentane extracts from used tubes (Table 1, experiment 4) over pentane extracts from unused tubes. Analysis of pentane and methanol extracts of previously occupied tubes by gas chromatography-mass spectrometry showed the presence of the same long-chain hydrocarbons found on the wasp cuticle, while high-performance liquid chromatography (RP-HPLC) analysis of methanol extracts showed five principal peaks identified as peptides (see Supplemental data). These peptides were found in the venom and on the cuticle.

The two most abundant peptides have been sequenced by mass spectrometry (specifically MALDI TOF/TOF) and named dominulins [4]. We divided the methanol extract into three fractions by HPLC: fraction 1 contained compounds eluted before the peptides; fraction 2 contained only the five peptides; and fraction 3 contained compounds eluted after the peptides [4]. Wasps did not show any preference for the residues from the fractions 1 or 3, but they significantly preferred the tubes treated with fraction 2 (peptide fraction) over pure evaporated methanol (Table 1, experiments 5–7). We also performed the same bioassays using methanol solutions of the two dominulins synthesised in the laboratory [4]. Once again, we observed a striking preference for the dominulin treated tubes over those treated with methanol only (Table 1, experiment 8).

Proteins and peptides are involved in communication in many vertebrates and aquatic invertebrates [1]. For example, in mice major urinary proteins, beside being carriers of volatile pheromones, are themselves cues mediating several aspects of social interactions [5]. An

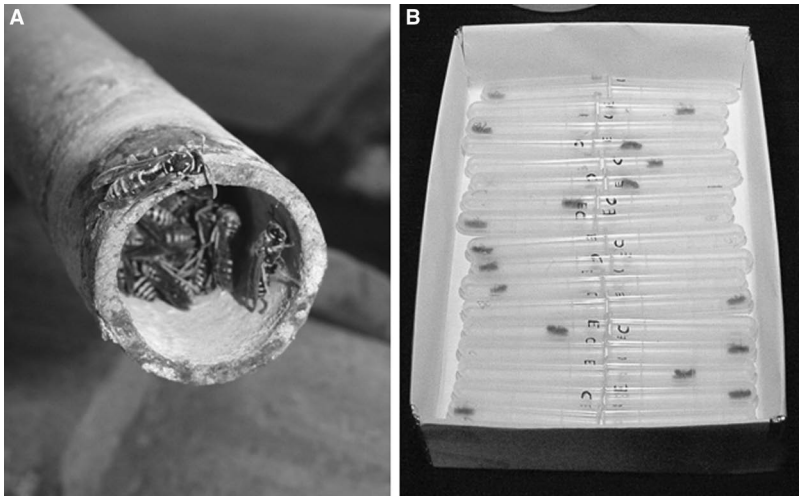

Figure 1. Peptide pheromones of hibernating *Polistes* wasps.

(A) A cluster of hibernating female *Polistes dominulus*. (B) The simple double choice experimental apparatus.

Table 1. Results from the behavioural tests with hibernating gynes of *Polistes dominulus*.

Expt	N	Treatment tube	Treat	Control tube	Contr	Test (Wilcoxon test)
1	98	Where 10 conspecific females had remained for 24 hours the previous day	79	Clean	16	Z= –6.799, P<0.001
2	18	As in Expt. 1 but the tubes were kept at room temperature and tested 8 months later	14	Clean	2	Z= –3.333, P=0.001
3	29	Contained evaporated MeOH extract from tubes treated as in Expt. 1	24	Evaporated MeOH	1	Z= –4.394, P<0.001
4	55	Contained evaporated pentane extract from tubes treated as in Expt. 1	15	Evaporated pentane	30	Z= –1.583, P=0.113 N.S.
5	69	Contained evaporated HPLC fraction of peptides collected from MeOH extract, from tubes treated as in Expt. 1	43	Evaporated MeOH	19	Z= –3.317, P=0.001
6	35	Contained evaporated HPLC fraction eluted before the peptide fraction in Expt. 5	10	Evaporated MeOH	19	Z= –1.873, P=0.061 N.S.
7	37	Contained evaporated HPLC fraction eluted after the peptide fraction in Expt. 5	15	Evaporated MeOH	18	Z= –1.080, P=0.280 N.S.
8	70	Contained an evaporated solution of synthetic Dominulins A e B	47	Evaporated MeOH	14	Z= –3.891, P<0.001

N refers to the number of wasps singly tested. Each wasp was observed six times in the one hour experiment. Treat and Contr refer, respectively, to the number of wasps recorded more times in the treatment or control tubes; the remaining wasps were observed three times in the treatment tube and three times in the control tube. With Wilcoxon signed ranks test, we compared the number of times that each wasp was scored in treatment versus control tubes.

interesting parallel with our findings can be drawn with peptide pheromone usage by barnacles and mussels to mark aggregation sites [6,7]. But among terrestrial insects, only peptides affecting female reproductive behaviour released with sperm by *Drosophila* males, [8] and cockroach epicuticular proteins (perhaps pheromone carriers) have been shown to be involved in chemical communication [9]. In social insects, there is some evidence for a possible peptidic pheromone in fire ant venom, but the substance was not identified [10].

In contrast, there is abundant evidence that cuticular lipids, in particular hydrocarbons, which given their low volatility act as contact pheromones, have important roles in colony organization [1]. But cuticular lipids cannot regulate wasp hibernacula choice, because we showed that pentane extracts containing these compounds are not attractive. Conversely, peptides elicited a strong and long-lasting attraction. The rather hydrophilic nature of dominulins implies that they can persist only in waterproof sites and, compared to hydrophobic lipids, are probably better indicators of well-sheltered quarters. Moreover, these peptides have an antibacterial activity [4] that might provide some added protection during over-wintering. This is the first definitive report of a peptidic pheromone in social insects, strongly suggesting that dominulins are cues indicating the location of safe hibernacula from one generation to the next.

Supplemental data

Supplemental data are available at http://www.current-biology.com/cgi/content/full/16/14/R530/DC1/

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